

Partial Characterization of Newly Synthesized Proteoglycans Isolated from the Glomerular Basement Membrane

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ABSTRACT Kidneys were perfused with [³⁵S]sulfate for 4 h *in vitro* to radiolabel sulfated proteoglycans. Glomeruli were isolated from the labeled kidneys, and purified fractions of glomerular basement membrane (GBM) were prepared therefrom. Proteoglycans were extracted from GBM fractions by use of 4 M guanidine-HCl at 4°C in the presence of protease inhibitors. The efficiency of extraction was ~55% based on ³⁵S radioactivity. The extracted proteoglycans were characterized by gel-filtration chromatography (before and after degradative treatments) and by their behavior in dissociative CsCl gradients. A single peak of proteoglycans with an *M_r* of 130,000 (based on cartilage proteoglycan standards) was obtained on Sepharose CL-4B or CL-6B. Approximately 85% of the total proteoglycans were susceptible to nitrous acid oxidation (which degrades heparan sulfates), and ~15% were susceptible to digestion with chondroitinase ABC (degrades chondroitin-4 and -6 sulfates and dermatan sulfate). The released glycosaminoglycan (GAG) chains had an *M_r* of ~26,000. Density gradient centrifugation resulted in the partial separation of the extracted proteoglycans into two types with different densities: a heparan sulfate proteoglycan that was enriched in the heavier fraction ($\rho > 1.43$ g/ml), and a chondroitin sulfate proteoglycan that was concentrated in the lighter fractions ($\rho < 1.41$). The results indicate that two types of proteoglycans are synthesized and incorporated into the GBM that are similar in size and consist of four to five GAG chains (based on cartilage proteoglycan standards). The chromatographic behavior of the extracted proteoglycans and the derived GAG, together with the fact that the two types of proteoglycans can be partially separated in the density gradient, suggest that the heparan sulfate and chondroitin sulfate(s) are located on different core proteins.

Latticelike networks of anionic sites have recently been detected in the laminae rara interna and externa of the glomerular basement membrane (GBM) (1, 15) (see Fig. 1). Based on cytochemical (16) and biochemical (17, 24) data, these sites have been shown to consist in large part of heparan sulfate, a sulfated glycosaminoglycan (GAG). Evidence has also been obtained indicating that GAGs play an important functional role in maintaining the charge selective permeability properties of the GBM to molecules, because their enzymatic removal was shown to result in increased permeability of the GBM to native ferritin (18) and to ¹²⁵I-labeled bovine serum albumin (20).

Up to now, our attention has been focused on establishing the nature and function of the GAGs present in the GBM. However, we assumed from the outset that the GAG chains were present as protein-polysaccharide complexes or proteoglycans covalently bound (by *O*-glycosidic linkages) to protein, because normally (with the possible exception of hyaluronic acid) GAGs do not exist free in the extracellular matrix (3, 25). Furthermore, that the sites bore a striking resemblance (after

staining with ruthenium red) to proteoglycan granules in other locations (12) and are sensitive to proteolytic digestion (16) suggested their proteoglycan nature.

In this study we set out to extract and to partially characterize the heparan sulfate proteoglycan from purified GBM, using established techniques for the isolation of proteoglycans (9). Because the amount of GAG present in the GBM is small (<1% of its dry weight [17]), we labeled the proteoglycans with [³⁵S]sulfate in a kidney perfusion system to facilitate their isolation and characterization by monitoring radioactivity. The results, which were briefly presented elsewhere (19), demonstrate that two types of newly synthesized proteoglycans can be recovered from purified GBM: heparan sulfate proteoglycan, which is the predominant type (~85%), and a chondroitin sulfate proteoglycan (~15%).

MATERIALS AND METHODS

Materials

Male, Charles River CD^R rats weighing 240–300 g were used in these experi-

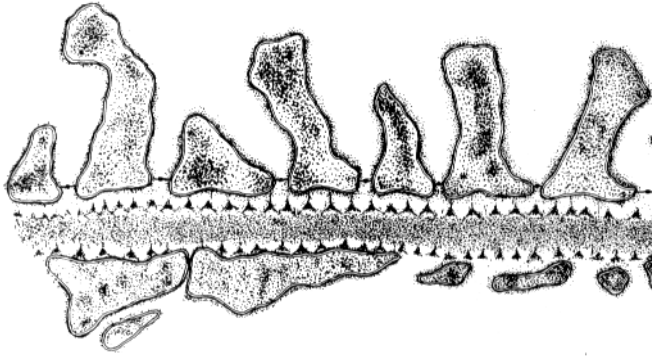


FIGURE 1 Diagrammatic representation of anionic sites in the lamina rara interna and externa of the GBM based on results of labeling with cationic probes. The sites are visualized as angular particles (~20 nm) with fine filaments (~3 nm) extending from their points to connect one particle with another, with the membranes of adjoining endothelial and epithelial cells, and with the lamina densa.

ments. Chondroitinase-ABC was purchased from Miles Laboratories, Inc., Elkhart, Ind.; papain (twice crystallized) from Sigma Chemical Co., St. Louis, Mo.; the zwitterionic detergent, Zwittergent 3-12 from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; [^3H] serine (2.75 Ci/mmol) and [^{35}S]sulfate (100 Ci/mmol) from New England Nuclear, Boston, Mass. Sepharose and Sephadex were obtained from Pharmacia Inc., Piscataway, N. J. and Hydrofluor scintillation fluid from National Diagnostics, Inc., Advanced Applications Institute Inc., Parsippany, N. J. [^3H]Serine-labeled cartilage proteoglycan monomer (21) was a gift of Dr. James Kimura, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

Column Chromatography

Columns of Sepharose CL-4B and CL-6B (110 \times 0.9 cm) were eluted with 4 M guanidine-HCl, 100 mM Tris-HCl, 0.1 M sodium sulfate, 0.5% (vol/vol) Triton X-100, pH 7.0. Their flow rates were ~2.0 ml/h, and effluent fractions of 0.5 ml were collected. The void (V_0) and total (V_t) volumes of the columns, were determined by eluting [^3H]serine-labeled cartilage monomer (for V_0) and [^3H]serine (for V_t). PD-10, Sephadex G-25 columns were eluted with the same buffer, and 0.25-ml fractions were collected (21).

Radioisotope Analysis

Each effluent fraction (0.25 or 0.5 ml) in 4 M guanidine-HCl was diluted with 0.7 ml of 70% ethanol, and 12.5 ml of Hydrofluor were added. Samples were counted in a Beckman LS 355 scintillation counter (Beckman Instruments, Inc.). The spillover in samples with both ^3H and ^{35}S activities was corrected by using an external standard.

Kidney Perfusion

Animals were anesthetized with pentobarbital, the right kidney was isolated, and the renal artery was cannulated and flushed with Krebs-Ringer bicarbonate (KRB). The cannulated kidney was removed from the animal, and kidney perfusion was carried out in a recirculating perfusion system as described by DeMello and Maack (7). The main modification involved perfusion of kidneys at a relatively lower flow rate (12–15 ml/min, instead of 38–40 ml/min). The perfusate consisted of modified KRB containing [^{35}S]sulfate (100 $\mu\text{Ci}/\text{ml}$). The final inorganic sulfate concentration of the perfusate was adjusted to 50 μM (5.8 $\mu\text{g}/\text{ml}$). The kidneys were labeled for up to 4–5 h under normothermic conditions. The incorporation of the precursor [^{35}S]sulfate was linear up to 4 h, so this time-point was chosen to achieve maximal labeling of proteoglycans. After 4 h, the kidneys were perfused for an additional 30 min with a chase medium containing ~5 mM inorganic sulfate. Tissue samples were taken and processed for electron microscopy. The remaining portions of the labeled kidneys were then immersed in 0.15 M NaCl, rapidly frozen, and kept at -20°C overnight.

Isolation of Glomeruli and Basement Membranes

Kidneys were thawed to 4°C and glomeruli were isolated and GBM prepared

therefrom by detergent treatment as previously described (15). A small fraction of the labeled GBM was processed for autoradiography; the remaining GBMs (from 18 rat kidneys) were pooled. 1-ml aliquots (two or three per kidney) of a GBM suspension were prepared and kept at -20°C until further use.

Extraction of [^{35}S]Sulfate-labeled Proteoglycans from the GBM

Each frozen aliquot of GBM was thawed to 4°C , and proteoglycans were extracted with 1 ml of 4 M guanidine-HCl containing 0.01 M sodium EDTA, 0.01 M sodium acetate, pH 5.8, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine-HCl and 0.001 M phenylmethylsulfonyl fluoride (28). The extraction was carried out for 24 h at 4°C with stirring. The unextracted residue was pelleted by centrifugation at ~10,000 rpm in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and saved for further processing. A small aliquot of the supernate was used for monitoring the radioactivity and for calculating total counts per minute in the extract. The remaining supernate containing the extracted proteoglycans was stored frozen until further analysis.

Fractionation and Characterization of the Extracted Proteoglycans

The extracts were thawed (to 4°C) and chromatographed on PD-10 columns (to remove free sulfate). Aliquots (0.5 ml) of the material that eluted in the void volume (>95% of the total counts) were then chromatographed on Sepharose CL-4B and CL-6B as described above; effluent fractions were monitored for radioactivity. Approximately 85% of the radioactivity applied to these columns was recovered in the effluent fractions. Other aliquots of the extracts were extensively dialyzed against distilled water, lyophilized to dryness, and divided into two portions. One portion was digested with chondroitinase-ABC (0.25 U/ml) in 0.1 M Tris-HCl buffer for 2 h at 37°C (30), while the other was treated with nitrous acid (prepared by mixing equal volumes at 20% (wt/vol) butyl nitrite and 1 N HCl) for 4 h at room temperature (2). Both the enzyme-digested samples and the nitrous acid-treated samples were re-lyophilized to dryness. They were then reconstituted with 0.5 ml of 4 M guanidine-HCl, mixed with V_0 and V_t markers and chromatographed on Sepharose CL-6B and CL-4B. In separate experiments, aliquots of the GBM extract were successively digested with chondroitinase-ABC followed by nitrous acid treatment before chromatography. The molecular weight of the GBM proteoglycans was estimated by interpolation of K_{av} values for peak elution positions with the data obtained from tryptic-chymotryptic digests of cartilage proteoglycan (14).

Characterization of GBM Proteoglycans in Dissociative CsCl Density Gradient

The GBM extracts were adjusted to an initial density of 1.42 g/ml by addition of 0.45 g of CsCl/g solution, centrifuged at 33,000 rpm in a SW 50.1 rotor for 72 h at 10°C (10), and fractionated into five approximately equal fractions, D_1 to D_5 , from bottom to top. The density of each fraction was determined with a Bausch & Lomb Abbé-3L refractometer (Bausch & Lomb Inc., Scientific Optical Products Div., Rochester, N. Y.). Aliquots of each of the fractions were chromatographed on Sepharose CL-4B before and after treatments with chondroitinase-ABC or nitrous acid, as described above.

Characterization of GBM GAGs

GAGs were obtained from two sources: (a) either directly from the GBMs labeled with [^{35}S]sulfate; or (b) from 4 M guanidine-HCl extracts of the GBM. In the latter case, aliquots were extensively dialyzed against distilled water and lyophilized. GAG chains were released from both sources either by treatment with 0.5 N NaOH at 45°C for 1 h (28) or by digestion with papain (1 mg/ml) in 0.1 M sodium acetate buffer, pH 5.5, 5 mM cysteine, and 5 mM sodium EDTA for 6 h at 60°C (17). Subsequently, both the papain- and alkaline-treated samples were extensively dialyzed against distilled water, lyophilized, and chromatographed before and after treatment with chondroitinase-ABC or nitrous acid, on Sepharose CL-6B. The molecular weights of the chains were estimated by comparison of the peak elution positions with the values obtained by Wasteson for chondroitin sulfate (33).

Characterization of Unextracted GAG Remaining in the GBM

The unextracted residue (remaining after guanidine extraction) was hydrolyzed with 0.5 N NaOH at 50°C overnight (to release GAG), and the total counts

released were determined. Aliquots of the hydrolysate were extensively dialyzed against distilled water and lyophilized to dryness. The dried residue was reconstituted as described above and then chromatographed on PD-10 and Sepharose CL-6B, before and after treatment with chondroitinase-ABC or nitrous acid.

RESULTS

Preservation of Glomeruli and of the Labeled GBMs

The overall architecture of the glomeruli was well preserved. The basement membranes prepared by the detergent method consists of intact loops of GBM (see Fig. 19 of reference 15). Autoradiography (not shown) revealed numerous silver grains associated with them. The efficiency of labeling of the GBM with [³⁵S]sulfate in the organ perfusion system was ~100-fold that observed in vivo (24). The rate of incorporation of the precursor into proteoglycans extracted from the GBM was linear up to 4 h; at this time point an average of ~40,000 cpm were present in the GBM fraction prepared from each kidney of a mature rat weighing 250–300 g (Table I).

Extraction of Newly Synthesized Proteoglycans from the GBM

Initially, several different procedures were tested for extraction of the proteoglycans from the GBM. Maximum efficiency of 55% was achieved when GBM were extracted with 4 M guanidine-HCl (Table I). The presence of Zwitterionic detergent in combination with guanidine-HCl did not improve the efficiency of extraction, although this procedure has proved to be effective for extracting proteoglycans from chondrocytes (22). More than 95% of the radioactivity in the 4 M guanidine-HCl extract was recovered in the void volume of a PD-10 column, indicating that the radioactivity was present in the newly synthesized, macromolecular components of the GBM.

Chromatographic Analyses of the Extracted Proteoglycans

When the proteoglycans (extracted with 4 M guanidine-HCl) were chromatographed on Sepharose CL-4B or CL-6B, they eluted as a single peak of radioactivity with K_{av} values of 0.45 and 0.23, respectively (Fig. 2A and B). When the extracted proteoglycans were subjected to nitrous acid oxidation, a procedure that selectively degrades *N*-sulfated GAGs (heparan sulfates) (2), ~88% of the radioactivity was released from the proteoglycan peak into the total column volume, whereas when the extracted proteoglycans were digested with chondroitinase ABS (which specifically degrades chondroitins 4- and 6-sulfate and dermatan sulfate), before chromatography, 12–15% of the radioactivity was released (Fig. 3). In both cases, the elution position of the resistant fractions (peaks C and B, respectively, in Fig. 3) was unchanged. Successive treatments with chondroitinase-ABC and nitrous acid resulted in release of 100% of the radioactivity from the extracted proteoglycans. These results demonstrate that the majority of the proteoglycan macromolecules are sensitive to nitrous acid and resistant to chondroitinase-ABC treatment, indicating that they contain heparan sulfate. A minority of the proteoglycan molecules are resistant to nitrous acid and sensitive to digestion with chondroitinase ABC, indicating that they contain chondroitin-4 or -6 sulfate or dermatan sulfate. Based on their similar elution profiles, both populations of proteoglycans appear to have virtually the same size distribution.

TABLE I
Efficiency of Extraction of [³⁵S]Sulfate-labeled Proteoglycan from the GBM* (Data Are Given per Kidney)

Extraction procedure	Total cpm in the residue		% Extraction
	Total cpm in the extract	Total cpm in the residue after hydrolysis†	
4 M GuCl for 24 h at 4°C	25,400	20,760	55
4% Zwittergent for 24 h at 4°C	4,120	39,170	10
4% Zwittergent for 12 h followed by 4 M GuCl for another 12 h at 4°C	25,740	20,890	55
4% Zwittergent plus 4 M GuCl for 24 h at 4°C	24,310	20,840	54

* Radiolabeled basement membranes were extracted, and the residue subsequently was hydrolyzed in alkali; both the extract and the hydrolysate were chromatographed on PD-10 Sephadex G-25 columns where >95% of the radioactivity in each case was recovered in the V_0 fraction. Total counts in the GBM preparation from one kidney varied between 30,000–47,000 cpm. † Alkaline treatment (0.5 N NaOH) at 50°C for 12 h.

Behavior in Dissociative CsCl Gradients

When the extracted proteoglycans were fractionated in a dissociative CsCl density gradient, a partial separation of the two types of proteoglycans was achieved (Table II). Although the separation was incomplete, the nitrous acid-sensitive proteoglycans were concentrated in the fractions of higher density (i.e., D_1 and D_2 with $\rho > 1.43$ g/ml), whereas the chondroitinase-sensitive proteoglycans were concentrated in the D_4 and D_5 fractions at buoyant densities < 1.41 g/ml. When aliquots of the gradient fractions were chromatographed on Sepharose CL-4B in each case there was a single peak with a minor increase in the K_{av} values between D_1 ($K_{av} = 0.44$) and D_5 ($K_{av} = 0.49$). This indicates that the proteoglycans in the bottom fractions were slightly larger than those in the top fractions.

Characterization of GAGs of the GBM

When alkaline hydrolysates of whole GBMs or of the 4 M guanidine-HCl extracts were chromatographed on Sepharose CL-6B, there was a shift in the elution profiles of the released GAG chains as compared with those of the intact proteoglycans (Fig. 4). The K_{av} increased from 0.23 (for the intact proteoglycan) to 0.44 (for the GAG chains). Treatment of GAGs with nitrous acid or chondroitinase-ABC before chromatography resulted in the release of ~85 and ~15%, respectively, of the radioactivity into the total column volume. In either case there was no detectable change in the elution profile of the resistant GAG peak (Fig. 5, profiles B and C).

The GAG fractions obtained after papain digestion (before or after degradative treatments) had profiles nearly identical to those obtained for GAGs recovered after alkaline hydrolysis.

GAGs in the Unextracted GBM Residue

The GAG fractions released by alkaline hydrolysis from the unextracted GBM residue were very similar to those released from the extracted proteoglycans in (a) their elution profile on Sepharose CL-6B and (b) in the fact that ~85% of the GAG fraction was sensitive to nitrous acid, whereas ~15% was sensitive to chondroitinase-ABC (data not shown). This indicates that the GAG composition of the unextracted proteoglycans is similar to that of the extracted proteoglycans.

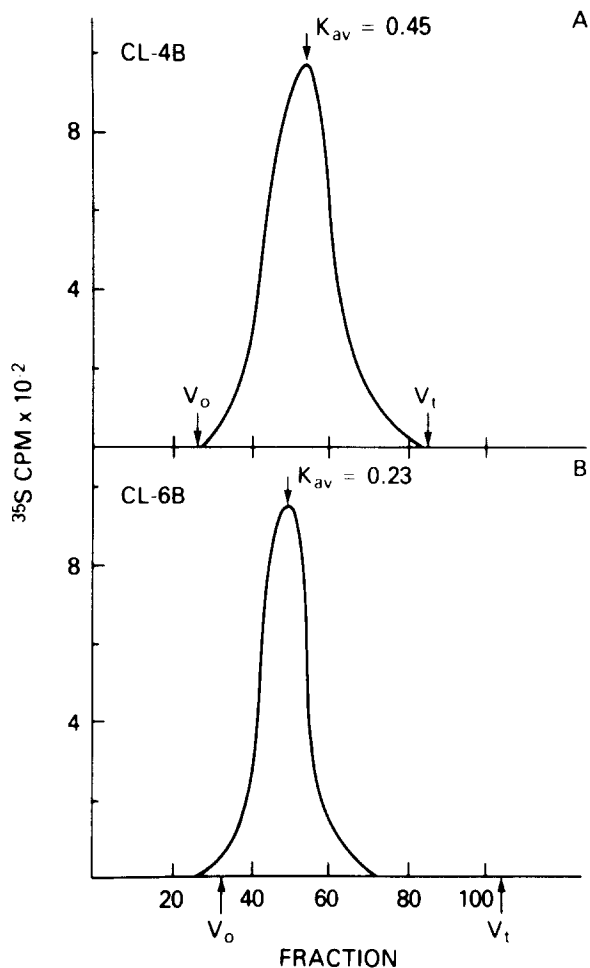


FIGURE 2 Sepharose CL-4B (A) and CL-6B (B) chromatograms of aliquots of a 4 M guanidine-HCl extract of GBM. Arrows indicate where the ^3H -labeled molecular weight standards (cartilage proteoglycan monomer [V_o] and serine [V_t]) that were included in each run elute. An approximate molecular weight of 130,000 for the intact proteoglycan is estimated by interpolating the K_{av} values for peak elution positions with the data obtained from the enzymatic digests of cartilage proteoglycan (14).

DISCUSSION

The results of this investigation demonstrate that proteoglycans are synthesized and incorporated into the GBM during perfusion of rat kidneys *in vitro*. Two types of newly synthesized, sulfated proteoglycans were extracted from GBM fractions: a nitrous acid-sensitive heparan sulfate proteoglycan (HS-PG) and a chondroitinase-ABC-sensitive, chondroitin sulfate proteoglycan (CS-PG). The major component was heparan sulfate proteoglycan, which made up ~85% of the total. It should be noted that the relative amounts of the heparan sulfate and chondroitin sulfate proteoglycans synthesized and incorporated into the GBM *in vitro* is identical to that of radiolabeled heparan sulfate and chondroitin sulfate found to be synthesized and incorporated into the GBM *in vivo* (24).

The size of the two types of proteoglycans (as determined by the elution profiles on Sepharose CL-6B) appear similar and is equivalent to that of a chondroitin sulfate-peptide fraction (tryptic digest) isolated from cartilage (14) with an average M_r of 130,000, containing four or five GAG chains. However, the two proteoglycans could be partially separated by virtue of their behavior in a dissociative CsCl gradient, where the HS-

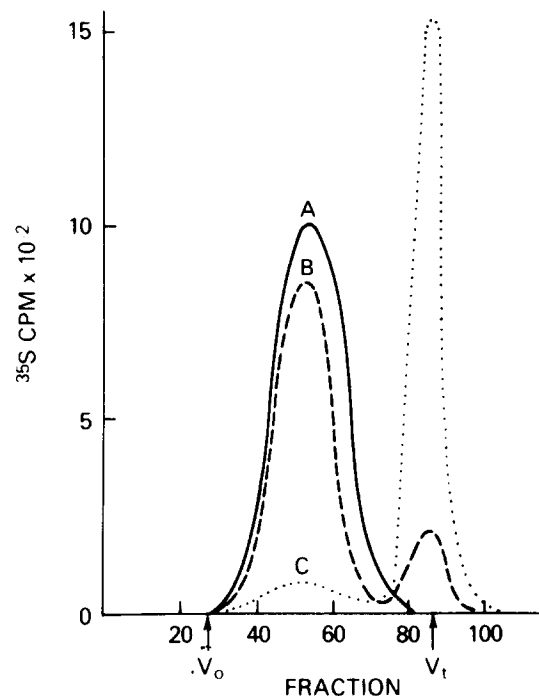


FIGURE 3 Sepharose CL-4B chromatograms of newly synthesized proteoglycan either intact (A) or after treatment with chondroitinase-ABC (B) or nitrous acid (C). Treatments with chondroitinase-ABC and nitrous acid released ~12% and ~88% of the radioactivities, respectively, to the total column volume.

TABLE II
Distribution of Newly Synthesized Radiolabeled Intact Proteoglycans in a Dissociative CsCl Gradient*

Fraction	Density g/ml	Total cpm %	% Total cpm HS-PG	% Total cpm CS-PG	K_{av} (Sepharose CL-4B)
D ₁	1.47	30	30	0	0.44
D ₂	1.44	21	20	1	0.45
D ₃	1.42	16	14	2	0.46
D ₄	1.41	15	11	4	0.48
D ₅	1.39	18	10	8	0.49

* Glomerular basement membranes from one ^{35}S -labeled kidney were extracted with 4 M guanidine-HCl, and the extract was fractionated by centrifugation on a CsCl gradient under conditions described in the Materials and Methods. Five fractions (D₁-D₅) were collected and subjected successively to digestion with chondroitinase-ABC to identify chondroitin sulfate proteoglycans (CS-PG) and nitrous acid oxidation to identify heparan sulfate proteoglycans (HS-PG).

PG was enriched in the heavier fraction ($\rho > 1.43$) and the CS-PG was concentrated in the lighter fraction ($\rho < 1.41$). In general, proteoglycans with higher buoyant densities have a higher ratio of GAG to protein (9); however, the degree of sulfation can also influence the density. Even though the separation of the two proteoglycans is incomplete in the gradient, the results strongly suggest that the two types of GAG are present on two different core proteins. Moreover, the fact that after nitrous acid or chondroitinase-ABC treatment of the proteoglycans the elution positions of the resistant fractions do not change, renders it unlikely that there is any appreciable portion of the proteoglycans that contains both chondroitin sulfate and heparan sulfate chains.

Regarding the location of the two types of proteoglycans, we have previously presented cytochemical evidence based on

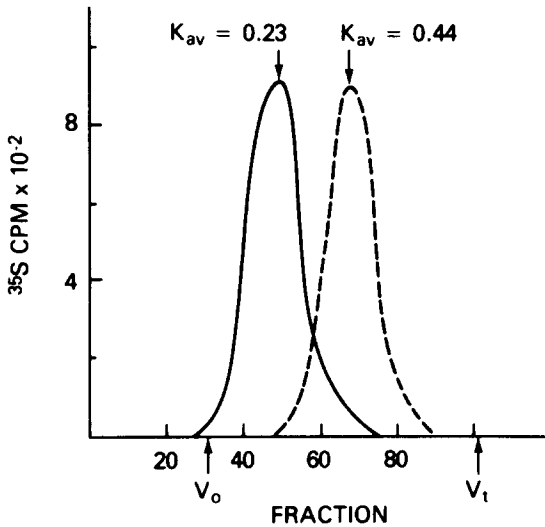


FIGURE 4 Sepharose CL-6B chromatograms of intact proteoglycan (solid line) and of GAG chains released after alkaline treatment (dashed line). With alkaline treatment, the K_{av} increased from 0.23 (~130,000 mol wt) to 0.44 (~26,000 mol wt), indicating that the newly synthesized proteoglycan has four to five GAG chains attached to its core protein.

specific enzyme removal that the heparan sulfate proteoglycans are concentrated in the laminae rarae (interna and externa) of the GBM (16), where they are most likely the products of the overlying endothelium and epithelium, respectively. In addition, they are also found in the mesangial matrix. No direct information is available on the location in the glomerulus or the cell source of the chondroitin sulfate proteoglycans; however, Striker et al. (31) have recently analyzed GAG synthesized by glomerular cell lines in culture, and have shown that cultured epithelial cells synthesize predominantly heparan sulfates, whereas mesangial cells synthesize predominantly chondroitin sulfates. These findings raise the possibility that the CS-PG may be the product of the mesangial cells.

It is clear from the present results as well as our previous studies on the GAG composition of the rat (17, 24) and dog (26) GBM that the HS-PG is the main proteoglycan component of the GBM. We have also established that the presence of these GAG (as well as chondroitin sulfates) is required for the GBM to carry out its normal filtration function (18, 20). Accordingly, the properties of these molecules and their constituent polysaccharide moieties have great inherent interest. Based on our results it appears that each heparan sulfate monomer contains several GAG chains, because the intact heparan sulfate proteoglycan elutes on the column much earlier ($K_{av} = 0.23$) than the released GAG. We have noted above that the elution profile of the HS-PG (as well as the CS-PG) is similar to that of a chondroitin sulfate-peptide fraction ($M_r = 130,000$) that contains four or five GAG chains (each with an average M_r of 26,000) obtained from cartilage. Thus, the HS-PG isolated from the GBM differs from those isolated from other sources. To date heparan sulfate proteoglycans have been isolated from only a few sources—notably hepatocyte (29) (or hepatoma [27]) cell membranes or the basement membrane-like matrix secreted by a mouse tumor (EHS sarcoma) (11). The former is much smaller (~75,000) and has smaller GAG chains (~14,000), whereas the latter has a much larger M_r (~750,000) and larger GAG chains (~70,000).

Up until recently, heparan sulfate and heparan sulfate pro-

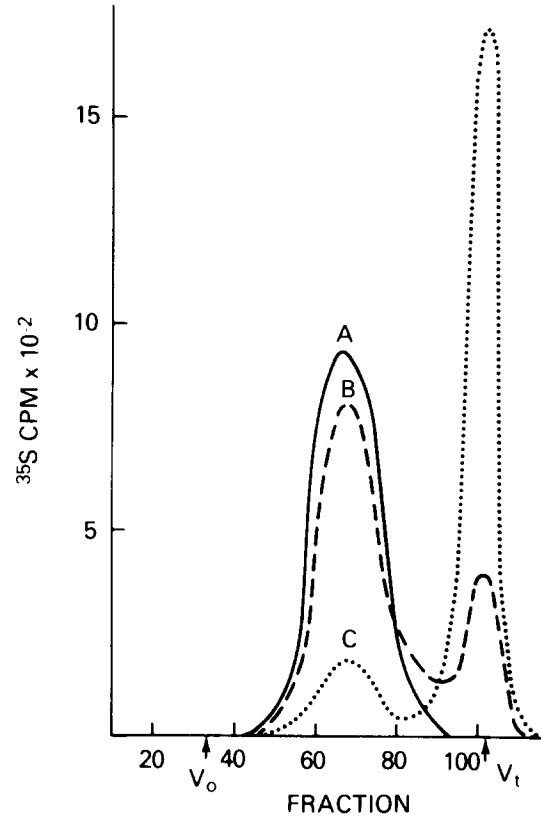


FIGURE 5 Sepharose CL-6B chromatograms of GAGs obtained after alkaline hydrolysis of proteoglycans and eluted either directly (A), or after chondroitinase-ABC digestion (B), or after nitrous acid treatment (C). Treatments with chondroitinase-ABC and nitrous acid released ~15 and ~85% of the radioactivities, respectively, to the total column volume. Note that the proportion of radioactivities released by these treatments is similar to that released from intact proteoglycans by the same treatments (Fig. 3).

teoglycans were believed to be associated primarily with cell surfaces (3, 25) where they are known to interact with fibronectin and other cell surface components (6, 34). Our studies have demonstrated that heparan sulfate proteoglycans are widely distributed among renal basement membranes, because we have found ruthenium red-stained proteoglycan granules that are sensitive to digestion with heparitinase associated with the basement membranes of the renal tubule epithelium, the vascular endothelium of the peritubular capillaries, and Bowman's capsule (16). Earlier, regular networks of proteoglycan granules had been seen after staining with ruthenium red in association with embryonic basement membranes (13, 32) and those of mouse mammary epithelium (8). Thus, proteoglycans are widely distributed among basement membranes in a variety of locations and may represent a general component of basement membranes.

Our results indicate that ~55% of the total proteoglycans synthesized by the perfused kidney and incorporated into the GBM over a 4-h incubation period in vitro can be extracted by the dissociative solvent, 4 M guanidine-HCl, whereas ~45% cannot. It is of interest that the GAG chains released from the extracted proteoglycans and those released from the insoluble residue are indistinguishable in their chromatographic behavior and composition. It can be assumed that the former proteoglycan fraction is noncovalently, but firmly, associated with the GBM. Although the proteoglycans remaining in the residue appear to be the same or very similar to those solubilized, they

have formed a tighter, possibly covalent, interaction with other components of the GBM. Whether the soluble and insoluble proteoglycans represent different compartments or whether, with time, more of the extractable proteoglycans become insoluble remains to be determined. Indeed the interactions by which these proteoglycans associate with other components of the GBM, such as basement membrane collagen or laminin or fibronectin, are not known at present. They may involve interactions via either the polypeptide or GAG portions of the proteoglycans or most likely both. It is of interest to note that our immunocytochemical studies (4, 5) indicate that the attachment proteins fibronectin and laminin, like the HS-PG, are both concentrated in the laminae rarae, whereas type IV collagen is concentrated in the lamina densa. Recent studies on heparan sulfate proteoglycans from hepatocytes (23) and cells in culture (6) indicate that heparan sulfate interacts with polysaccharide receptors on the cell surfaces, and also indicates that at least some of the HS-PG molecules interact with the cell membrane through hydrophobic portions of their core protein (23).

Based on their properties, a few possible functions have been suggested for the HS-PG and the CS-PG present in the GBM, of which the most important is their role in glomerular permeability (18, 20). Under normal conditions the GBM is highly negatively charged, which is important for retaining (by electrostatic repulsion) anionic plasma proteins in the circulation. Direct evidence for a role of GAG in GBM permeability was obtained by demonstrating that enzymatic removal of GAG results in an increased permeation of anionic proteins (ferritin [18] and albumin [20]) into the GBM, and leakage of these tracers into the urinary spaces.

In summary, we have isolated proteoglycans from purified GBM fractions and have partially characterized the proteoglycans synthesized and incorporated into the GBM *in vitro*. We have shown that there are two types of proteoglycans present—a heparan sulfate proteoglycan and a chondroitin sulfate proteoglycan—and we have obtained evidence that the two types of GAG are present on two different core proteins. Further work is needed to clarify the nature of the interaction of the proteoglycans with other GBM components and with the surface components of glomerular cells.

This work was supported by research grant AM 17724 (to M. G. Farquhar) from the National Institutes of Health.

Received for publication 24 April 1981.

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